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Genetic And Temporal Variation In Resource Exchange Between An Ectomycorrhizal Fungus, Rhizopogon Occidentalis, And Seedlings Of Pinus Radiata

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GENETIC AND TEMPORAL VARIATION IN RESOURCE EXCHANGE BETWEEN AN ECTOMYCORRHIZAL FUNGUS, RHIZOPOGON OCCIDENTALIS, AND SEEDLINGS OF PINUS RADIATA

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ABSTRACT

Various economic theories have been adapted to explain the evolutionary persistence of resource exchange mutualisms such as mycorrhizae. The ratio of resources exchanged is an important variable common to all these models. Measuring resource exchange ratios is the first step in testing the hypotheses predicted by these models and will provide insight into resource dynamics of mutualisms. We examined how the carbon, nitrogen and phosphorus resource exchange dynamics between *Pinus radiata* and an ectomycorrhizal fungus, *Rhizopogon occidentalis*, varied between native populations of *Pinus radiata* and over the first 64 weeks of the mycorrhizal mutualism. Using a mycocosm approach, the C:N and C:P exchange ratios were determined by comparing the amount of C respired by fungi and C assimilated in fungal biomass to the amount of N and P assimilated in plant tissues over a time period. Resource exchange was assessed at 8, 16, 32, and 64 weeks after inoculation for two *Pinus radiata* native populations. Resource exchange increased over time during the development of the mycorrhizal mutualism. Pine seedlings from the Cambria population transferred greater amounts of carbon to the ectomycorrhizal fungus, with the majority of this carbon being respired by fungal biomass. The ratio of resources exchanged did not vary over time or between pine populations, pointing to total resource fluxes as the potential mechanism behind changes in mycorrhizal fungal compatibility between populations of host plants.

LIST OF ABBREVIATIONS OR SYMBOLS

C	Carbon
N	Nitrogen
P	Phosphorus

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INTRODUCTION

Mutualisms are one of the most common interactions between species seen in nature. They can be found in all ecosystems around the globe, aquatic and terrestrial, and involve almost all species either directly or indirectly. Furthermore, mutualisms and their impacts span all levels of biological organization, from cells to ecosystems, and occur as widespread interactions, such as pollinator/plant mutualisms, or in unique situations, such as those found along marine hydrothermal vents (Bronstein 2009, Chaston and Goodrich-Blair 2010). However, scientists struggle to explain the evolutionary stability of these relationships (Hoeksema and Bruna 2000). Some evolutionary theory predicts that because the partners of a mutualism will act in accordance with their own benefit, the relationship will inevitably disintegrate, as evolution favors traits that allow one of the partners to reap the benefits of the interaction while avoiding the costs associated with providing the favorable services to the other partner (Bronstein 2009). Nevertheless, mutualisms continue to be conserved and ubiquitous throughout evolutionary time.

Various theoretical approaches have been modified from the field of economics to explain the establishment of mutualisms and to predict the conditions that are most favorable for their persistence (Akçay 2015). Borrowing ideas from cooperative game and bargaining theories, Akçay and Roughgarden proposed a model based on negotiation between the partners of a mutualism (2007). They suggested that partners develop continuous negotiations in the form of contracts, which can be accepted or rejected by the other partner. Over time, these negotiations reach

equilibrium in the form of the Nash Bargaining Solution, which maximizes the product of the partners' benefits from the symbiosis. Similarly, Kummel and Salant developed a model involving partner choice to predict the coexistence of multi-specific mutualistic partner communities (2006). In this situation, the host either switches between partner species or selectively adds partners in order to reduce the marginal costs of the mutualism. Finally, Schwartz and Hoeksema employed the ideas of comparative advantage and specialization to create a model for the stability of resource-exchange mutualisms (1998). This model indicates that partners benefit when they specialize in acquiring a single resource and then trade for the other resource. They further expanded their model to show how resource requirements of the partners can influence the success of the mutualism (Hoeksema and Schwartz 2003). McGill extended these models of comparative advantage with game theory to predict the evolution of beneficial mutualisms (McGill 2005). Subsequent research has also combined the ideas of bargaining theory and comparative advantage to predict resource specialization, exchange ratios, and variation in mutualism outcomes (Grman et al. 2012).

Although these models differ in the economic principles they employ and the predictions they make, they share one common variable: the exchange ratio. In the case of a resource-exchange mutualism, the exchange ratio is the amount of one resource exchanged by a partner to obtain a certain amount of another resource from the other partner. The exchange ratio appears as a variable in all of the aforementioned models. In Akçay and Roughgarden's bargaining model, the exchange ratio is the equilibrium point reached after continuous negotiations: the Nash Bargaining Solution (2007). Kummel and Salant included the exchange ratio as r_j, which is used in the exchange function unique to each mycorrhizal fungus engaging in a mutualism with a host plant (2006). The exchange ratio is pivotal in developing the exchange function and marginal cost curves used to determine partner choice in their model. In Schwartz and Hoeksema's relative advantage models, the exchange ratio takes the form of the slope of the trade acquisition isocline, which represents all possible

combinations of two resources that mutualistic partners can acquire with specialization and trade. They specifically identified the exchange ratio as the variable *T*, the trade cost ratio (Schwartz and Hoeksema 1998, Hoeksema and Schwartz 2003). The exchange ratio is foundational to all of these models and therefore should be measured as a first step in testing these mutualism models.

The mycorrhizal mutualism provides an ideal system with which to take this first step. Mycorrhizae are resource-exchange mutualisms between plant roots and hyphal fungi (Smith and Read 2008). In this symbiosis, the plant trades photosynthetically-fixed carbon compounds belowground to mycorrhizal fungi at the root interface in exchange for other nutrients, primarily nitrogen and phosphorus, mobilized from the soil by the fungi (Smith and Read 2008). This mutualism provides a unique opportunity to measure exchange ratios because it involves the transfer of resources, which are discrete and quantifiable. In addition, the mycorrhizal symbiosis occurs in a short time scale, making measurements of exchange ratios over the development of the mutualism feasible. More importantly, mycorrhizal associations are ecologically important and ubiquitous. Mycorrhizae occur with most terrestrial plants and can have strong influences on plant diversity, nutrient cycling, community dynamics, and ecosystem responses to global change (Trappe 1987, van der Heijden and Sanders 2002).

Previous research has investigated the exchange of carbon for nitrogen or phosphorus in the mycorrhizal mutualism. Some studies have focused on carbon allocation and fluxes of nitrogen or phosphorus and how this varies with time (Jones et al. 1991, Colpaert et al. 1996), fungal species (Colpaert et al. 1996, Jones et al. 1998), plant species (Qu et al. 2004), amount of nutrient supply (Douds et al. 1988, Colpaert et al. 1996), or mycorrhization (Douds et al. 1988). These studies distinguish between above- or belowground carbon partitioning; however, they do not parse out the amount of carbon allocated to the fungi specifically versus carbon retained in the roots. This

limitation prevents these studies from being used to determine exchange ratios between the partners. Additionally, other research has determined specific fates of carbon partitioning belowground, but without accounting for belowground respiration (Pearson and Jakobsen 1993, Kiers et al. 2011), or without simultaneously measuring nitrogen or phosphorus exchange (Andersen and Rygiewicz 1995, Fransson et al. 2007). On the other hand, several studies have traced the exchange of carbon for nitrogen and/or phosphorus between plants and mycorrhizal fungi using isotope pulses (Ek 1997, Bidartondo et al. 2001, Kiers et al. 2011). These studies are useful to estimate short-term exchange ratios, but not cumulative exchange ratios needed to test the economic models of mutualisms.

The resource exchange dynamics of the mycorrhizal mutualism may be influenced by other factors besides resource exchange ratios. The supply of carbon from host plant to partner fungi has been shown to depend on the plant's own nitrogen demand (Corrêa et al. 2008, 2011, 2012) and the availability of nutrients in the soil (Corrêa et al. 2006, 2011, Kiers et al. 2011) and can, in turn, influence the supply of nutrients back to the plant (Kiers et al. 2011). As such, the exchange of carbon may not be determined by the supply of nutrients (i.e. the exchange ratio). Quantifying total volumes of resources exchanged between plants and fungi will elucidate which factor is most important in determining the mycorrhizal resource exchange dynamics.

Resource exchange in the mycorrhizal mutualism should be influenced by the supply and demand for carbon and nutrients by both partners (Akçay 2015). Specifically, the plant's supply of carbon may impact both the total volume of trade and the exchange ratio. Carbon production by the plant is directly linked to its photosynthetic rate and other factors, such as respiration and plant structure (Le Roux et al. 2001, Landsberg and Sands 2011). Such physiological traits of plants are partly genetically determined and have been found to differ between populations or genotypes of *Pinus* species (Marshall et al. 2001, Hoeksema and Thompson 2007, Rowell et al. 2009, Lüttge et al.

2011, Hoeksema et al. 2012). Furthermore, genetic variation within and between populations of *Pinus* species influences establishment and function of the ectomycorrhizal symbiosis with particular fungal species. For example, *P. muricata* genotypes and geographically isolated populations of *P. radiata* vary in their compatibility with multiple *Rhizopogon spp.* (Hoeksema and Thompson 2007, Piculell et al. 2008, Hoeksema et al. 2012). Also, genotypes of *P. pinaster* associating with *Rhizopogon roseolus* differed in shoot N concentration, magnitude of their growth response to mycorrhizal inoculation, and the degree of mycorrhizal colonization (Sousa et al. 2012). Thus, mycorrhizal nutrient exchange ratios have the potential to vary between different populations and genetic families of pine trees.

Over the course of development of the mycorrhizal mutualism, the partners' supply and demand for carbon and nutrients will fluctuate, potentially influencing the exchange ratios. For example, plants experience seasonal changes in growth and biomass allocation, with an increase in belowground carbon allocation and root growth at the end of the growing season (Smith and Read 2008). Mycorrhizal fungi also have temporal variability in their carbon demand. The production of hyphal networks or sporocarps will produce a large carbon demand from mycorrhizal fungi on the plant partner. These temporal changes may influence the mycorrhizal exchange ratios. Jones et al. (1991) found that the phosphorus acquisition efficiency of ectomycorrhizal willows varied over time, with a greater efficiency early in the development of the mutualism. They attributed the decline in efficiency seen later in the experiment to a higher carbon demand from the ectomycorrhizal fungus in order to build non-absorbing structures, such as ectomycorrhizal mantles, rhizomorphs, and reproductive structures. Additionally, the production of hyphal networks may impact the exchange ratios at the beginning of the mutualism. As mycorrhizae are initially developing, the fungi will require large amounts of carbon to establish a hyphal network, while initially providing a low supply

of nutrients to the plant. In other words, the plant may experience a 'start-up cost' with the mycorrhizal mutualism.

Monterey Pine (*Pinus radiata* D. Don) provides a unique opportunity to explore the influence of genetic variation on exchange ratios in the mycorrhizal mutualism. *P. radiata* occurs as a native plant in five discrete populations along the Pacific coast of North America: three on the coast of central California (Año Nuevo, Monterey, Cambria), and two Mexican island populations off the coast of Baja California (Guadalupe Island, Cedros Island). As a result of their geographic isolation, these populations have diverged genetically, evolving variation in traits that may influence the exchange ratios of their mycorrhizal mutualisms (Rowell et al. 2009, Hoeksema et al. 2012). The populations have been found to differ in their growth patterns, specifically seasonal basal area increment (Rowell et al. 2009), mass, specific root length, and root:shoot ratio (Hoeksema et al. 2012). Two populations, Cambria and Cedros Island, tend to fall at opposite ends of the spectrum. Cambria seedlings have more mass and allocate a greater proportion of their biomass to producing coarse roots, whereas Cedros Island seedlings have less mass, allocate nearly equal amounts of biomass to shoot and roots, and produce finer roots (Hoeksema et al. 2012).

In addition, the *P. radiata* populations have diverged in their compatibility with a particular ectomycorrhizal fungal taxon: *Rhizopogon*. *Rhizopogon* is a ubiquitous genus of ectomycorrhizal fungi that associates with a variety of genera in the Pinaceae family (Molina and Trappe 1994). It has been shown to positively influence the growth, performance, and uptake of P, K, Na, and NH_3 of inoculated seedlings, as well as provide tolerance to drought, pathogens and heavy metals (Molina and Trappe 1994, Sousa et al. 2014). *Rhizopogon* is used to inoculate pine seedlings before outplanting into plantations and has also been found to establish in containerized pine seedlings in nurseries and colonize trees in young pine plantations (El Karkouri et al. 2002, 2005, Steinfeld et al. 2003). In

addition, *Rhizopogon* is an abundant ectomycorrhizal fungus in native populations of pines (Taylor and Bruns 1999, Kjøller and Bruns 2003, Rusca et al. 2006, Hoeksema et al. 2012, Gehring et al. 2014, Garcia et al. 2015, Glassman et al. 2015).

While *Rhizopogon roseolus* (Corda) Th. Fries and *Rhizopogon occidentalis* Zeller and Dodge are common associates with *P. radiata* seedlings of Californian populations, both of the Mexican island populations of *P. radiata* have evolved reduced compatibility with *R. roseolus* (Hoeksema et al. 2012) and the Cedros Island population in Mexico has evolved reduced compatibility with *R. occidentalis* (Hoeksema and Thompson 2007). This observed pattern may be related to variation in resource exchanges between the fungi and these different populations of plants. *Rhizopogon* has a long-distance, high-biomass exploration type with an extensive hyphal network and rhizomorphs (as defined by Agerer 2001). This high carbon demand may drive up either exchange ratios or total volumes of trade, making the association less favorable for either or both partners under some circumstances. This may eventually lead to selection for reduced compatibility and/or altered patterns of resource exchange over generations. These hypotheses are supported by the drier, hotter climate experienced on Cedros Island compared to that of the Californian populations. With reduced water availability, Cedros Island seedlings may experience selection against association and/or for reduced C:N and C:P exchange ratios with a high biomass fungal type such as *Rhizopogon*. Alternatively, genetic drift may be the cause of the changes in compatibility, i.e., random genetic changes in the isolated Mexican island populations may have led to reduced mycorrhizal compatibility with *Rhizopogon* species.

The objectives of this research were to measure the magnitudes of resource exchange and resource exchange ratios between the ectomycorrhizal fungus, *Rhizopogon occidentalis*, and seedlings of Monterey pine, *Pinus radiata*, to determine how resource exchange differs between the native

populations of *P. radiata* in Cambria, CA and Cedros Island, Mexico, and to assess how resource exchange changes over time. I hypothesized that *R. occidentalis* offers less favorable resource exchange ratios (i.e., greater amounts of carbon traded for units of nitrogen and phosphorus) to genetic families of *P. radiata* from the Cedros Island population, compared to those from the Cambria population. Furthermore, I hypothesized that resource exchange ratios will vary over time, with greater resource exchange ratios (i.e., higher amounts of carbon traded for units of nitrogen and phosphorus) established during periods of increased fungal growth (e.g., during initial colonization of root tips and production of rhizomorphs). To test these hypotheses, I used a mycocosm approach to measure nutrient allocation to plant and fungal structures and processes in the *P. radiata*/*R. occidentalis* mycorrhizal mutualism. Specifically, amounts of carbon (C) transferred to *P. radiata* and amounts of nitrogen (N) and phosphorus (P) transferred to *R. occidentalis* were measured. From these measurements, I determined resource exchange ratios (C:N and C:P) for representative genetic families from the two populations of *P. radiata* at multiple time points during the symbiosis.

METHODS

Overview

This experiment used a mycocosm approach to measure the resource exchange ratios established between *P. radiata* and *R. occidentalis*. Seedlings of two genetic families from each of two native populations of *P. radiata* (Cambria, CA and Cedros Island, Mexico) were germinated in the lab, inoculated with spores of *R. occidentalis,* and grown in dual-chambered mycocosms. A total resource tracking approach was used to estimate total fluxes of C, N, and P as well as C:N and C:P resource exchange ratios over four time periods during the mycorrhizal mutualism. Measurements of C accumulated in fungal biomass were made using the fungal-specific chemical, ergosterol. Since it rapidly degrades after fungal tissue senesces, ergosterol was used as a proxy for only living biomass. Measurements of C released as fungal respiration were made using an infrared gas analyzer. Amounts of N and P assimilated in plant tissue were also measured. Total fluxes of C, N, and P and C:N and C:P exchange ratios were analyzed statistically to determine variation between pine populations and changes over time. My first hypothesis predicts that greater C:N and C:P ratios will be established between *R. occidentalis* and families of *P. radiata* from the Cedros Island population. My second hypothesis predicts that C:N and C:P ratios will be greater at the first (Week 1-8) and third (Week17-32) time periods and the ratios will be lower at the second (Week 9-16)and final (Week 33-64) time periods.

Seed collection and plant propagation

Pinus radiata (D. Don) is a coniferous plant species with five native populations. Three of these populations are located along the Pacific coast of central California, the southernmost of these Californian populations being located in Cambria (35°32'6.0" N, 121°4'48.0" W). The other two are island populations located off the coast of Baja California, the southernmost of these being the Cedros Island population (28°20'48.0" N, 115°13'24.0" W)(Hoeksema et al. 2012). Two *P. radiata* genetic families, specifically open-pollinated seed families, from each of the Cambria and Cedros Island populations were compared in this experiment: Cambria 2, Cambria 9, Cedros 2, and Cedros 13. Seeds were previously collected in 2006 from trees in the Cambria and Cedros Island populations (Hoeksema et al. 2012). The trees from which the two Cedros Island seeds families were collected are located in the southern of the two sub-populations on Cedros Island. As described by Hoeksema et al. (2012), cones were collected from trees adjacent to a randomly placed transect. 80-100 seeds of each of the four *P. radiata* families were surface-sterilized by soaking for two minutes in a 10% bleach solution. Seeds were thoroughly rinsed and then completely submerged in water for 48 hours. Seeds were kept moist at 4°C for 21 days and agitated daily to discourage mold growth. Seeds were then planted into autoclaved peat/vermiculite substrate (Metro-Mix 366; SunGro Horticulture Canada Ltd.; Seba Beach, Alberta, Canada) in sterile deep plug flats. Seeds/seedlings were allowed to germinate and grow for fourteen weeks in a Conviron Model ATC40 environmental chamber at 26°C with a 14-hr photoperiod (~302 μmol m^{-2} s^{-1}), receiving weekly de-ionized water sufficient to completely soak the soil.

Mycocosm construction

104 dual-chambered mycocosms were constructed of two parallel plates of clear polycarbonate (23 cm tall by 38 cm wide) separated by PVC spacers (2.5 cm thick), as shown in Figure 1. The growth volume is separated into two halves (approximately 1 L each) by a rigid divider routed to 90% openness, filled with sterile coarse sand, and sealed on both sides with 44 μm nylon mesh, which was known from previous testing in our laboratory to prevent passage of pine roots but allow passage of fungal hyphae. For control mycocosms, the center divider was sealed with 50 μm thick plastic to prevent any exchange between halves.

Figure 1: Experimental setup of CO_2 measurements on dual-chambered mycocosm. Dual-chambered mycocosm (red) with custom LI-COR chamber (yellow) seated on top with IRGA head (blue) attached to make soil CO_2 efflux rate measurements.

Field soil was collected in 2010 along a vegetational gradient from grassland to the interior of *P. radiata* forest at the Kenneth S. Norris Rancho Marino Reserve, Cambria, CA (Hennig 2011), passed through a 2mm sieve to remove coarse debris and manually homogenized. One liter of homogenized field soil was suspended in water and filtered to 5μm to create a microbial wash. The remaining field soil was diluted with coarse play sand, using 1 part soil, 4 parts sand, and approximately 800 ml of this soil mixture was added to each half of the mycocosms. Mycocosms were autoclaved at 121°C for 1 hour to sterilize the soil, sealed, and stored in growth chambers in a room with air filtered by a HEPA filter to reduce the likelihood of potential contamination from non-target fungi. Mycocosm covers were created from 50 μm thick black plastic to prevent algal growth in mycocosms.

Seedling inoculation and planting

Following a fourteen week germination and growth period, each seedling was removed from the soil plug and roots were washed free of soil. Seedlings were checked visually for ectomycorrhizal colonization. Because non-target ectomycorrhizal fungi were observed on the root systems of these seedlings, all dichotomously-branched mycorrhizal root tips were manually removed from the root systems to minimize subsequent abundance of non-target fungi on the seedlings.

As outlined in Table 1, two control groups and four treatment groups were established. (-) Microbe and (+) Microbe control mycocosms contained sterilized soil in both halves, a mesh divider sealed with plastic, and one seedling dipped in DI water planted into one side of each mycocosm. (+) Microbe control mycocosms had the addition of ten milliliters of microbial wash to both halves of each mycocosm. Six Cambria 2 and twelve Cambria 9 seedlings were randomly assigned to control mycocosms. Treatment mycocosms contained sterilized soil in both halves, a mesh divider,

one inoculated seedling planted into one side of each mycocosm, and ten milliliters of microbial wash added to both halves of each mycocosm. Twenty seedlings from each of the four genetic families were dip-inoculated with *R. occidentalis* spore slurry and planted into treatment mycocosms. Spore slurry for mycorrhizal inoculation was created by blending DI water with two *R. occidentalis* sporocarps, collected from the Kenneth S. Norris Rancho Marino Reserve, Cambria, CA (JDH 211: collected 1/18/2011, dried, used 30 months later; JDH 223: collected 12/16/2012, refrigerated, used ~6.5 months later). The spore concentration was adjusted to 2.066×10^7 spores/ml and refrigerated until use for seedling inoculation, approximately 18 weeks later.

Table 1: Design of experiment.

Experimental group	**CAMBRIA 2** ***+ fungi +microbes***	**CAMBRIA 9** ***+ fungi +microbes***	**CEDROS 2** ***+ fungi +microbes***	**CEDROS 13** ***+ fungi +microbes***	**(+)MIC CONTROL** ***- fungi +microbes***	**(-)MIC CONTROL** ***- fungi -microbes***
Harvest #1 (Week 9)	5 rep (4)	5 reps (3)	5 reps (4)	5 reps (4)	3 reps (1)	3 reps (2)
Harvest #2 (Week 17)	5 reps (5)	5 reps (0)	5 reps (3)	5 reps (5)	3 reps (1)	3 reps (1)
Harvest #3 (Week 33)	5 reps (4)	5 reps (2)	5 reps (3)	5 reps (3)	2 reps (0)	2 reps (0)
Harvest #4 (Week 65)	5 reps (5)	5 reps (1)	5 reps (4)	5 reps (4)	2 reps* (0)	2 reps* (0)

Numbers in parentheses represent number of replicates with live seedling at harvest
*One replicate filled with soil on one side; no pine seedling planted

Mycocosms were thoroughly watered prior to seedling planting. On a weekly basis throughout the experiment, mycocosms were randomized and received sufficient water to thoroughly wet the soil in all mycocosms. All mycocosms were labeled, covered, and maintained in the growth chamber at 26^0C with a 14-h photoperiod (~302 μmol m^{-2} s^{-1}).

Data collection

Prior to planting, a subsample of sterile soil was reserved from three control mycocosms to be analyzed for ergosterol, C, N, and P content. Subsamples of spore slurry, microbial wash and DI water were reserved to be analyzed for ergosterol, C, N, and P content. Five Cambria 4 and five Cedros 10 seedlings were dip-inoculated and root systems were immediately clipped and frozen for ergosterol, C, N, and P analysis. Prior to creating the spore slurry, one *R. occidentalis* sporocarp (JDH 223) was subsampled and established in culture on agar media. Subcultures of JDH 223 were analyzed for ergosterol and C content.

Harvests occurred 8, 16, 32, and 64 weeks after seedlings were planted into mycocosms. At each harvest, soil CO_2 efflux rate was measured from both sides of each harvested mycocosm using a LI-COR LI-6400XT (LI-COR Biosciences, Lincoln, Nebraska, USA) and a custom chamber as pictured in Figure 1. The shoot of the pine seedling was cut just prior to efflux rate measurements made on the pine side of a mycocosm. Therefore, efflux from the pine side of treatment mycocosms represents respiration by pine seedling roots, fungal biomass, and microbes added through the microbial wash; efflux from the non-pine side of treatment mycocosms represents respiration by fungal biomass and microbes added through the microbial wash. Efflux from the pine side of (+) microbe control mycocosms represents respiration by pine seedling roots and microbes added through the microbial wash; efflux from the non-pine side represents background respiration by the microbes added by the microbial wash. Efflux from the pine side of the (-) microbe control mycocosms represents pine seedling root respiration and efflux from the non-pine side would represent background respiration from the sterile soil. Hence, there should theoretically be no measurable efflux from the non-pine side of the (-) microbe control mycocosms. Following efflux rate measurements, mycocosms were destructively harvested. For Harvest 1, this occurred within

one week of efflux rate measurements; for the remaining harvests, it was done within one hour of efflux rate measurements. Samples of homogenized soil were taken from each side of the mycocosm and frozen until analysis for C, N, P, and ergosterol content. The remainder of the soil from each side and pine seedling shoots were dried in a drying oven at 65° C for 48 hours and weighed. Pine seedling shoots were processed for analysis of C, N, and P. Pine seedling root colonization rates were assessed and colonizing fungi were morphotyped and sampled for molecular identification to determine the proportion of root tips colonized by the target fungus, *R. occidentalis*, and to identify any non-target ectomycorrhizal fungi. Root systems were then frozen until processed for analysis of C, N, P, and ergosterol content. All C, N, P, and ergosterol analyses were conducted in the laboratory of Dr. Kevin Kuehn at the University of Southern Mississippi.

Molecular identification

All root tips of pine seedling root systems were counted and morphotypes of colonized ectomycorrhizal root tips were described and counted. Genomic DNA was extracted from sampled root tips using Extract-N-Amp Tissue Kit Extraction and Neutralization Buffers (Sigma-Aldrich, St. Louis MO, USA) using a modified protocol. Each root tip was submerged in 10 µl of Extraction buffer and cycled with these conditions: 65°C for 10 min, 95°C for 10 min, after which 30 µl of Neutralization buffer was added and DNA was diluted to 20% with 160 µl of PCR-grade water. DNA was amplified in 8 µl PCR reactions containing 4 µl of 2.0X Apex RedTaq PCR Master Mix (Genesee Scientific Corporation, San Diego, CA, USA), 0.4 µl of each 10µM fungal-specific primer: ITS-1F and ITS-4, 2.2 µl of PCR-grade water, and 1 µl of DNA extract. Incubations were cycled with the following conditions: initial denaturation at 94°C for 3 min, then 30 cycles of denaturation at 94°C for 45 s, annealing at 53°C for 45 s, and extension at 72°C for 60 s, with a final extension at

72°C for 10 min. DNA amplification success was confirmed with a 1% agarose gel. PCR products were cleaned enzymatically using 10 µl incubations containing 0.05 µl of 20,000U/ml Exonuclease 1 enzyme, 0.2 µl of 5000U/ml Antarctic Phosphotase enzyme (New England Biolabs, Ipswich, MA, USA), 4.75 µl of PCR-grade water, and 5 µl of PCR product. Incubations were cycled using these conditions: 37°C for 30 min, 80°C for 20 min, 4°C for 5 min. PCR products were sequenced using 10 µl reactions containing 0.4 µl of BigDye Terminator v3.1 Ready Reaction Premix, 1.8 µl of 5X BigDye Terminator v3.1 Sequencing Buffer (Applied Biosystems), 0.5 µl of 10µM fungal-specific primer ITS-5, 6.3 µl of PCR-grade water, and 1 µl of PCR product. Reactions were cycled with these conditions: initial denaturation at 96°C for 1 min, followed by 45 cycles of denaturation at 95°C for 20 s, annealing at 52°C for 20 s, and extension at 60°C for 4 min. Products were dried using a vacufuge at 45°C for 30 min and then shipped to the DNA Lab at Arizona State University for cleaning and sequencing.

LI-COR parameters

Soil CO_2 efflux rates were measured using a LI-COR LI-6400XT (LI-COR Biosciences, Lincoln, Nebraska, USA) and a custom chamber. The LI-6400 Soil CO_2 Flux System software configuration was used to provide accurate measurements of soil efflux rates by maintaining the soil-gas concentration gradient. With this "closed" method, a target level is set to the ambient CO_2 level and a delta value is selected. CO_2 is scrubbed out of the chamber until the concentration inside the chamber reaches a level equal to target minus delta. The software measurements are recorded as the CO_2 concentration inside the chamber rises above ambient to a level equal to target plus delta. This measurement cycle is repeated for a selected number of iterations. For this experiment, the target level was reassessed prior to measurements for each mycocosm, a delta level of 5ppm was selected

to accurately measure the low rates of efflux, and five measurement cycles were completed for each side of the mycocosm.

Data synthesis

For each mycocosm, the following measurements were taken: shoot C, N, and P contents, and shoot mass; root C, N, P, and ergosterol contents, and root mass; pine side soil C, N, P, and ergosterol contents, and CO_2 efflux rates; non-pine side soil C, N, P, and ergosterol contents, and CO_2 efflux rates. In addition, DI water, spore slurry, microbial wash, sterile soil, and ten dip-inoculated seedling root systems were analyzed for C, N, P, and ergosterol content.

These measurements were used to calculate average total resource transfers for each pine genetic family over each time period. Carbon transferred from plant to fungus has two major short-term fates: fungal biomass or fungal respiration. Nitrogen and phosphorus transferred from fungus to plant has two fates as well: shoot tissue or root tissue (Figure 2). Total resource transfers were used to calculate N and P exchange ratios by dividing the total amount of C transferred to the fungus by the total amount of respective nutrient. Data from treatment mycocosms that contained a dead pine seedling at harvest were removed from the data set and not included in analyses.

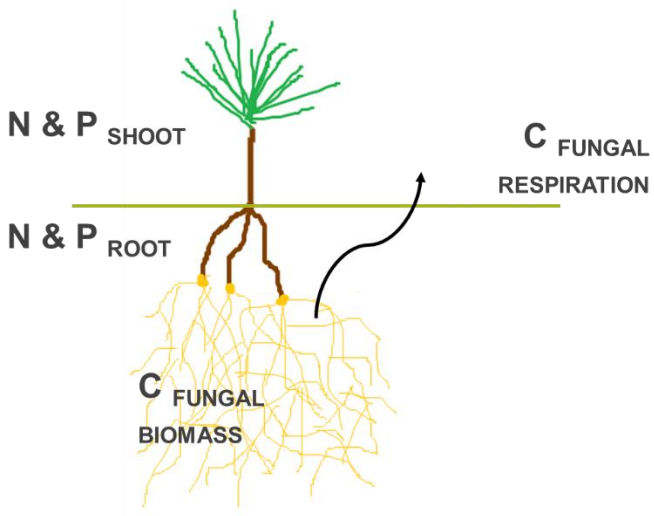

Figure 2: Fates of carbon, nitrogen, and phosphorus transferred between plant and fungus. Carbon transferred to the fungus can be incorporated into fungal biomass or respired by the fungus; nitrogen and phosphorus transferred to the plant can be incorporated into shoot or root biomass.

Estimation of carbon in fungal respiration ($C_{fungal\ respiration}$)

To determine the amount of C respired by fungal biomass, measurements of CO_2 respired per unit of living fungal biomass from the non-pine side of each mycocosm were used to estimate the total amount of fungal respiration from each mycocosm. Ergosterol was used as a proxy for living fungal biomass. Average soil ergosterol content per gram of soil for (+)MIC control non-pine sides (Figure 3, A) was used to represent the background amount of soil ergosterol present in the soil and microbial wash. This amount was multiplied by the soil mass and then subtracted from the total soil ergosterol content from each side of treatment mycocosms to calculate the experimental soil ergosterol content of each side (Figure 3, B_1 and B_2).

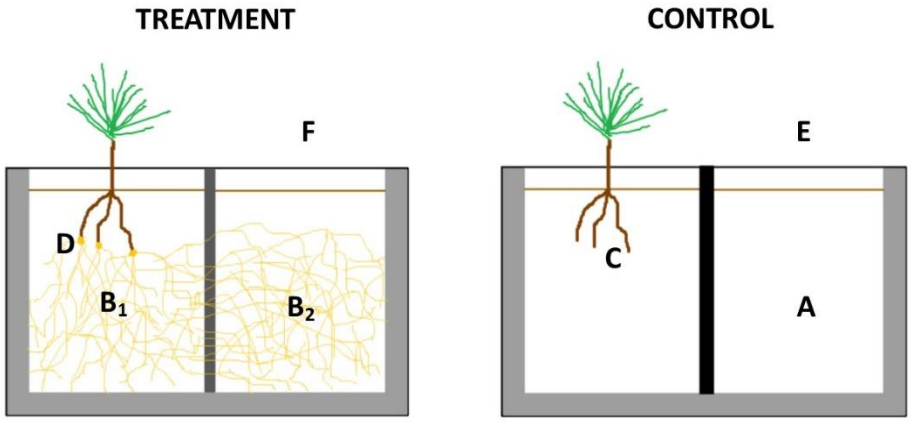

Figure 3: Calculating background ergosterol content. Diagram of background ergosterol content used in calculating amount of carbon in fungal respiration:

A = Average soil ergosterol content per gram of soil for (+)MIC control non-pine sides

B_1 = Experimental soil ergosterol content of treatment mycocosm pine side
= total treatment pine side soil ergosterol content − (treatment pine side soil mass * A)

B_2 = Experimental soil ergosterol content of treatment mycocosm non-pine side
= total treatment non-pine side soil ergosterol content − (treatment non-pine side soil mass * A)

C = Average root ergosterol content per dead root tip of (+)MIC control seedlings

D = Experimental root ergosterol content of treatment seedling
= total treatment seedling root ergosterol − (C * no. of treatment seedling dead root tips)

E = Average soil CO2 efflux rate from (+)MIC control non-pine sides

F = Experimental soil CO2 efflux rate of treatment mycocosm non-pine side
= total treatment non-pine side soil CO2 efflux rate − (E)

Pine root systems consisted of both *R. occidentalis*-colonized root tips and dead root tips. Saprobic fungi may decompose dead root tips, contributing to measurements of ergosterol. To calculate this background amount of ergosterol, the ergosterol content per dead root tip was averaged for (+)MIC control pine seedlings (Figure 3, C) and multiplied by the number of dead root tips present on each treatment pine root system. The background root ergosterol amount was subtracted from the total root ergosterol amount to calculate the experimental root ergosterol for

each treatment pine seedling (Figure 3, D). The experimental root ergosterol can also be considered the amount of ergosterol due to *R. occidentalis* on each root system.

To estimate background soil CO_2 efflux rate, the measured soil CO_2 efflux rate from the non-pine side of (+)MIC control mycocosms was averaged (Figure 3, E). This background rate of soil CO_2 efflux was subtracted from the total soil CO_2 efflux rate for each treatment mycocosm to produce the experimental CO_2 efflux rate (Figure 3, F). This quantity can also be considered the rate of CO_2 respiration of living *R. occidentalis* biomass present in the non-pine side of the mycocosm. If any of the experimental values for soil ergosterol, root ergosterol, or CO_2 efflux rate were negative, this indicated that there was no measureable presence of or respiration from *R. occidentalis* and values were set to zero.

To estimate the fungal CO_2 efflux rate from the pine side of each treatment mycocosm, the experimental CO_2 efflux rate from the non-pine side (Figure 3, F) was divided by the experimental soil ergosterol from the non-pine side for each mycocosm (Figure 3, B_2) to estimate rates of CO_2 efflux per unit *R. occidentalis* ergosterol. These rates were averaged for all treatment mycocosms, regardless of pine genetic family or harvest. The average CO_2 efflux rate per unit ergosterol was multiplied by the sum of the soil and root ergosterol contents from the pine side of each mycocosm (Figure 3, B_1 and D) to estimate the pine side experimental CO_2 efflux rate. Mycocosm experimental CO_2 efflux rate was calculated as the sum of the pine side and non-pine side experimental CO_2 efflux rates.

Mycocosm experimental CO_2 efflux rates were averaged for each pine genetic family at each harvest and averages were plotted over number of weeks and fitted with a least-squares logistic curve. Initial CO_2 efflux rates at Week 0 were set to 0 for all pine genetic families. To determine the total amount of CO_2 respired by *R. occidentalis* over each time period, the area beneath the curve was

calculated using Simpson's 1/3 method for integration. Curve fitting and integration were conducted using Python 2.7.6. The amount of CO_2 respired by the fungus during each time period was used with C in fungal biomass to calculate total C transfer from plant to fungus over each time period. Average rates of fungal respiration during each time period were also calculated by dividing the amounts of C respired by the fungus during each time period by the number of days during the respective time period.

Estimation of carbon in fungal biomass ($C_{\text{fungal biomass}}$)

The amount of C accumulated into fungal biomass over each time period was estimated using cumulative ergosterol content following the approach used by Hendricks et al. (2015) for ectomycorrhizal fungal mycelia production. Calculations of pine and non-pine side experimental soil ergosterol and experimental root ergosterol used in estimations of carbon in fungal respiration were summed to determine experimental ergosterol for each mycocosm. If any of the experimental values for soil ergosterol or root ergosterol were negative, this indicated that there was no measureable presence of *R. occidentalis* and values were set to zero. Mycocosm experimental ergosterol contents were averaged for each pine genetic family at each harvest. Cumulative experimental ergosterol content for each genetic family was calculated by summing the amount of ergosterol present at a particular harvest and the amounts of ergosterol present at all preceding harvests.

Cumulative experimental ergosterol contents were converted to amounts of C using ergosterol:biomass and biomass:C conversion factors developed from agar cultures of one *R. occidentalis* sporocarp (JDH 223) used in inoculation. Initial amounts of C in fungal biomass were set to 0 for all pine genetic families. The change in average amount of C in fungal biomass between two given harvests divided by the number of days during the time period represents the rate of C

transfer to fungal biomass during that time period. Negative rates of C transfer to fungal biomass were set to zero.

Carbon transferred to fungus (C_{fungus})

The total amount of C transferred from plant to fungus over each time period is equal to the sum of the amounts of C incorporated into fungal biomass and C released in fungal respiration over each time period ($C_{fungal\ respiration} + C_{fungal\ biomass}$). The total amounts of C transferred during each time period were divided by the total amount of N and P, respectively, to determine the resource exchange ratios (C:N and C:P). Also, rates of C transfer during each time period were calculated by dividing the total amount of C transferred during each time period by the number of days in each time period.

Nitrogen and phosphorus transferred to plant (N_{plant} and P_{plant})

To calculate the amount of N or P transferred from fungus to plant over each time period, the amount of these nutrients in the shoot and the root system of each pine seedling was measured. The total seedling nutrient contents were also averaged for each pine genetic family at each harvest. The difference between average seedling nutrient contents at two given harvests represents the amounts of nutrients transferred by the fungus to the plant during the time period. Negative rates of N and P transfer were set to zero. Total amounts of nutrient transfers over each time period were used with total amounts of C transfer to determine resource exchange ratios. Also, rates of nutrient transfers during each time period were calculated by dividing the total amount of nutrient transfer during each time period by the number of days in each time period.

In order to calculate initial seedling nutrient content, family-specific relative growth rates between Week 8 and Week 16 were used to extrapolate back and estimate initial shoot nutrient

content. Predicted initial shoot nutrient content was added to population-specific root nutrient content measured at planting to calculate initial seedling nutrient content.

Cambria 9 interpolation

All Cambria 9 seedlings pre-assigned to be harvested at Week 16 were dead and, consequently, no informative data were collected. Therefore, I fitted the data from the other three harvests with a linear fit and interpolated seeding N content, seedling P content, fungal biomass C amounts, and instantaneous fungal respiration rate at Week 16. These values were then used to estimate resource transfers between plant and fungus over each time period.

Statistical analysis

Univariate analysis of variance was used to determine the effect of time and pine population on resource fluxes between *P. radiata* and *R. occidentalis*. Separate ANOVA models including time (Week 0, 8, 16, 32, 64), pine population (Cambria, Cedros Island), and the interaction between time and pine population were used to analyze each of the following four response variables, which represent accumulations or instantaneous rates of resource exchange at the beginning of the experiment and at each of the four harvests: instantaneous fungal respiration rate (mycocosm experimental CO_2 efflux rates), C accumulation in fungal biomass (mycocosm C content of fungal biomass), N accumulation in seedlings (total seedling N content), and P accumulation in seedlings (total seedling P content). Separate ANOVA models including the main effects of time period (0-8 weeks, 8-16 weeks, 16-32 weeks, 32-64 weeks), pine population (Cambria, Cedros Island), and their interaction were used to analyze the following seven response variables, which summarize average rates or ratios of resource exchange during each of the four time periods of the experiment: rate of

C transfer to fungal respiration, rate of C transfer to fungal biomass, rate of C transfer to fungus, rate of N transfer to plant, rate of P transfer to plant, C:N exchange ratio, and C:P exchange ratio. Because these response variables are calculated using averages across replicates, the number of replicates for each pine population is reduced from a maximum sample size of 10 replicates to a sample size of 2 replicates, one for each pine family in each population. This, in turn, reduced the power of these statistical analyses.

To meet the assumptions of normality and homoscedasticity of model residuals, instantaneous fungal respiration rate, N accumulation in seedlings, and P accumulation in seedlings were log-transformed and C accumulation in fungal biomass was square root-transformed. For any significant main effects or interactions of the predictor variables, Tukey's HSD pairwise tests were used to determine the patterns in the response variable over time and between the two pine populations. Marginal means and standard errors were calculated to display the data graphically. Gross nutrient fluxes were also calculated by subtracting the maximum and minimum average values for each nutrient for each pine family over the length of the experiment. These gross nutrient fluxes and gross resource exchange ratios were interpreted visually since they were calculated using averages across replicates.

RESULTS

All *P. radiata* families were successfully inoculated with *R. occidentalis*, with an average colonization for all seedlings of 98.0% of available root tips (+/- 0.30 SE). No contaminant mycorrhizal fungi were observed on root systems at harvest or identified through Sanger sequencing of root tips. Table 2 contains values for calculations of C in fungal respiration outlined in Figure 3.

Table 2: Background ergosterol values. Estimated values of background ergosterol used in calculating amount of carbon in fungal respiration (see Figure 3)

Average soil ergosterol content per gram of soil for (+)MIC control non-pine sides (A)	0.096 ± 0.011 μg ergosterol/ g soil
Average root ergosterol content per dead root tip of (+)MIC control seedlings (C)	0.151 ± 0.058 μg ergosterol/ dead root tip
Average rate of CO_2 efflux from soil in (+)MIC control non-pine sides (E)	1.164 ± 0.358 μmol CO_2/m^2/ sec
Average rate of CO_2 efflux per unit *R. occidentalis* ergosterol (F/B_2)	0.105 ± 0.072 μmol $CO_2/m^2/sec/\mu g$ ergosterol

Nitrogen accumulation in *P. radiata* seedlings

The N content of *P. radiata* seedlings increased significantly over time, as expected with plant growth ($F_{4,49}$=11.714, p<0.001, Figure 4a). Seedlings from the Cambria population of *P. radiata* had a significantly greater N content than seedlings from the Cedros Island population ($F_{1,49}$=5.895,

p=0.019; Figure 4b). The two pine populations did not differ in their patterns of N accumulation over time (population x time interaction: $F_{4,49}$=1.152, p=0.343).

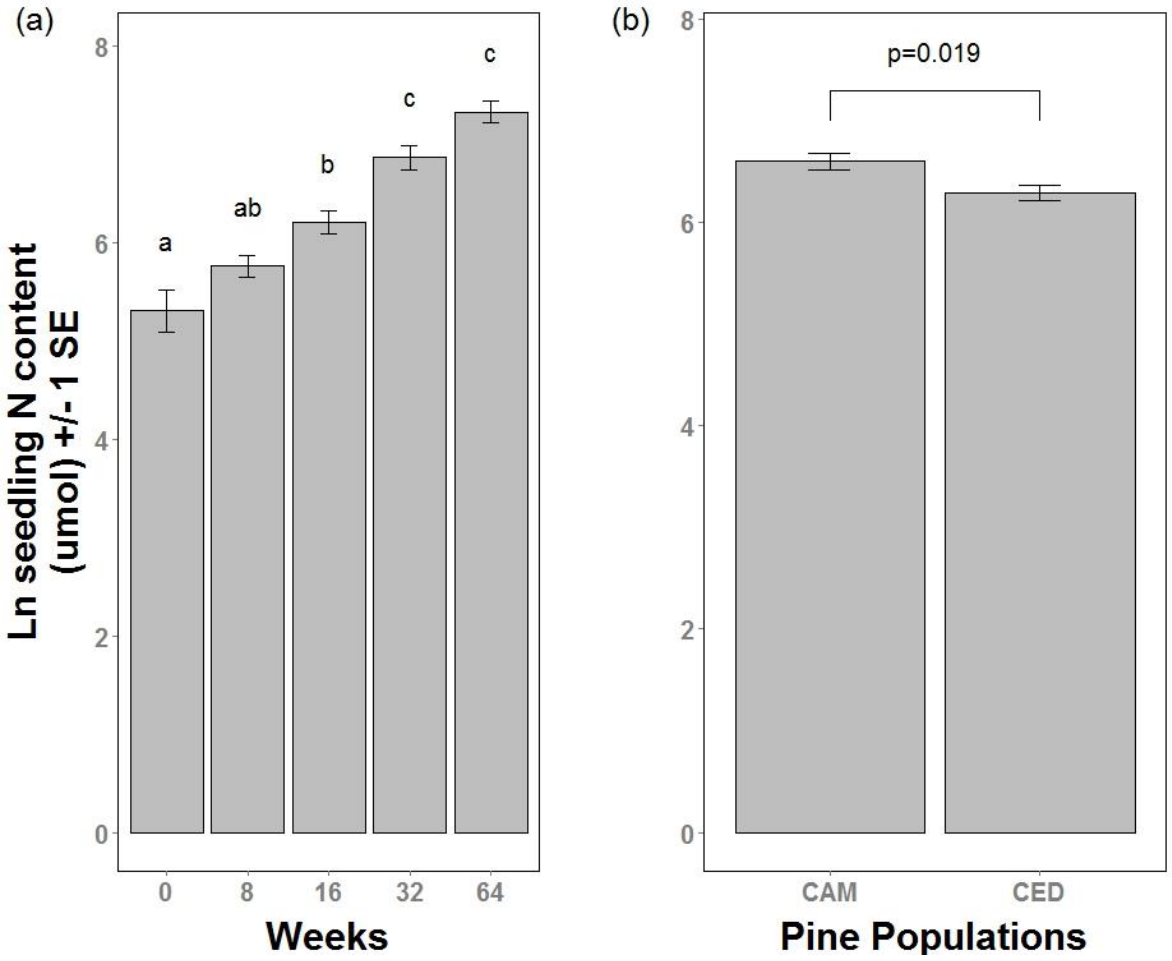

Figure 4: Seedling nitrogen content. (a) Nitrogen content of *Pinus radiata* seedlings increased significantly over time; (b) Cambria seedlings had significantly greater nitrogen content than Cedros Island seedlings. Marginal means and standard errors shown.

Phosphorus accumulation in *P. radiata* seedlings

The P content of *P. radiata* seedlings increased significantly over time, as expected with plant growth ($F_{4,49}$=15.582, p<0.001, Figure 5a). Seedlings from the Cambria population had significantly greater P contents than seedlings from the Cedros Island population ($F_{1,49}$=8.448, p=0.005; Figure 5b). The two pine populations did not differ in their patterns of P accumulation over time (population x time interaction: $F_{4,49}$=1.644, p=0.178).

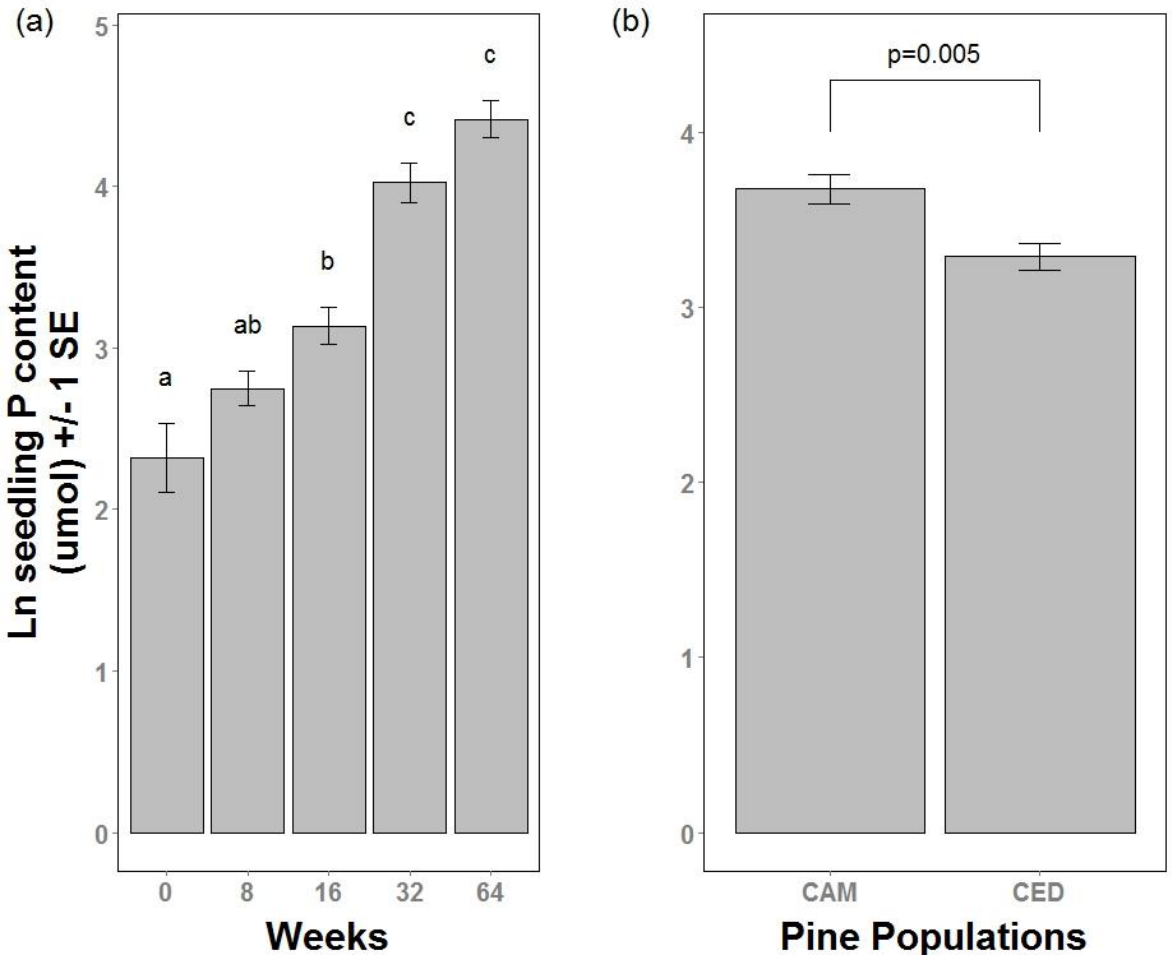

Figure 5: Seedling phosphorus content. (a) *Pinus radiata* seeding phosphorus content increased significantly over time; (b) *Pinus radiata* seedlings from the Cambria population had significantly higher phosphorus contents than seedlings from the Cedros Island population. Marginal means and standard errors shown.

Carbon accumulation in *R. occidentalis* biomass

Carbon accumulation in *R. occidentalis* biomass increased over time, as expected with fungal growth ($F_{4,10}$=24.607, p<0.001, Figure 6). *Rhizopogon occidentalis* associated with pine seedlings from either population did not differ in the C accumulation in fungal biomass ($F_{1,10}$=1.621, p=0.232). Also, the pattern of C accumulation in fungal biomass over time was not dependent on the pine population of the associated seedling (population x time interaction: $F_{4,10}$=0.431, p=0.784).

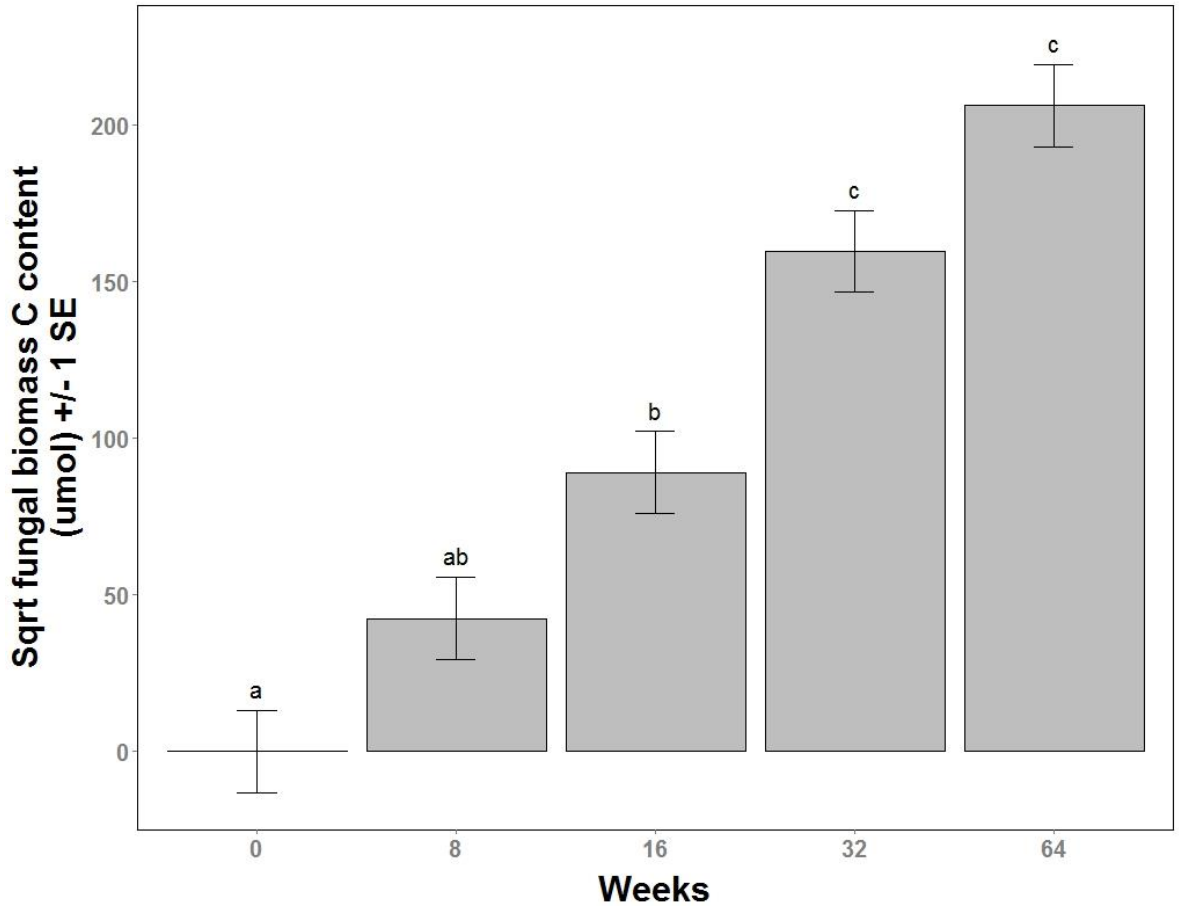

Figure 6: Fungal biomass carbon content. Accumulation of carbon in *Rhizopogon occidentalis* biomass increased significantly over time. Marginal means and standard errors shown.

Instantaneous respiration rate of *R. occidentalis*

The instantaneous rate of respiration by *R. occidentalis* increased significantly over time ($F_{4,49}$=38.055, p<0.001). The instantaneous fungal respiration rate increased significantly from the start to week 8, from week 8 to week 16, and from week 16 to week 32, followed by a plateau in instantaneous respiration rate through week 64 of the mutualism (Figure 7). However, the instantaneous rate of fungal respiration was not influenced by the pine population ($F_{1,49}$=0.114, p=0.737), and the two pine populations did not differ in how the instantaneous fungal respiration rate changed over time (population x time interaction: $F_{4,49}$=0.015, p=1.000).

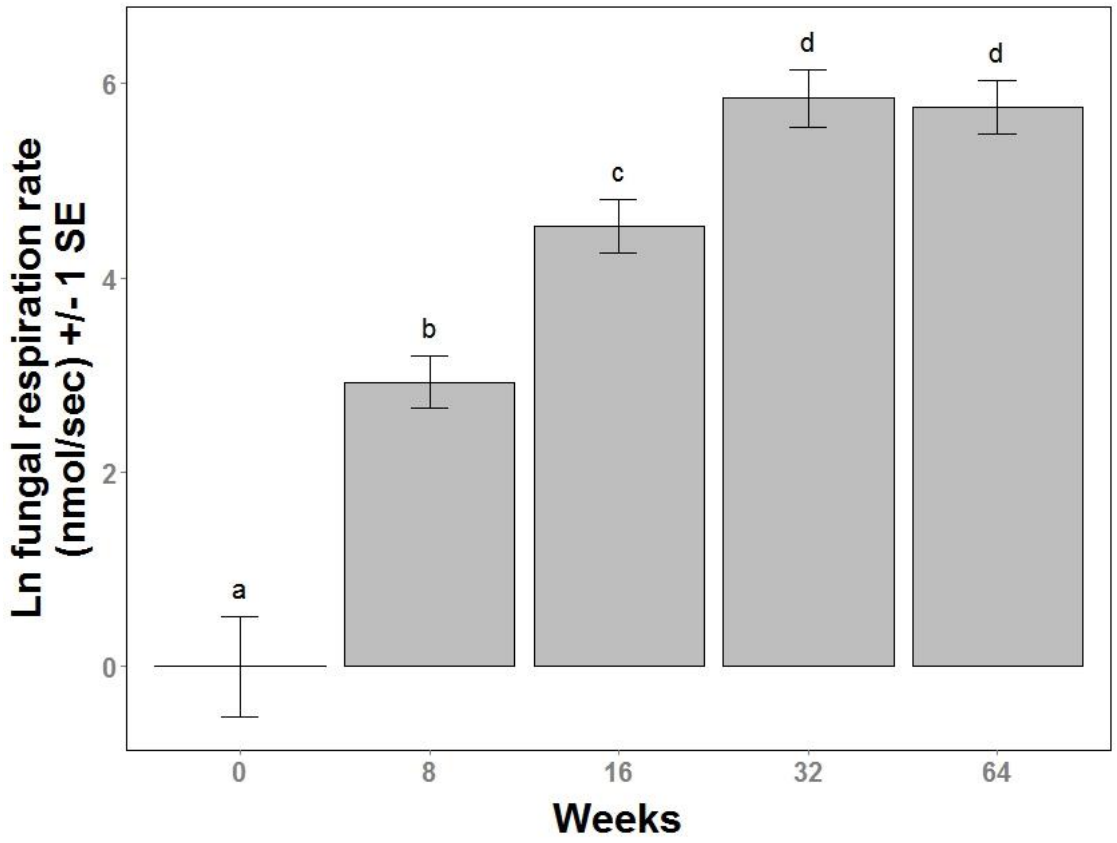

Figure 7: Instantaneous fungal respiration rate. *Rhizopogon occidentalis* instantaneous fungal respiration rate increased significantly over the first 32 weeks of the mutualism. Marginal means and standard errors shown.

Rate of nitrogen transfer to *P. radiata* seedlings from *R. occidentalis*

The rate of N transfer from *R. occidentalis* to *P. radiata* seedlings did not vary significantly between the two pine populations ($F_{1,8}$=1.093, p=0.326). However, the rate of N transfer did vary significantly over time, with increasing N transfer rates over the first 32 weeks of the experiment, followed by a large decrease in N transfer rate ($F_{3,8}$=4.455, p=0.040; Figure 8).The pattern of change in N transfer rates over time did not depend on pine population (time period x population interaction: $F_{3,8}$=2.500, p=0.134).

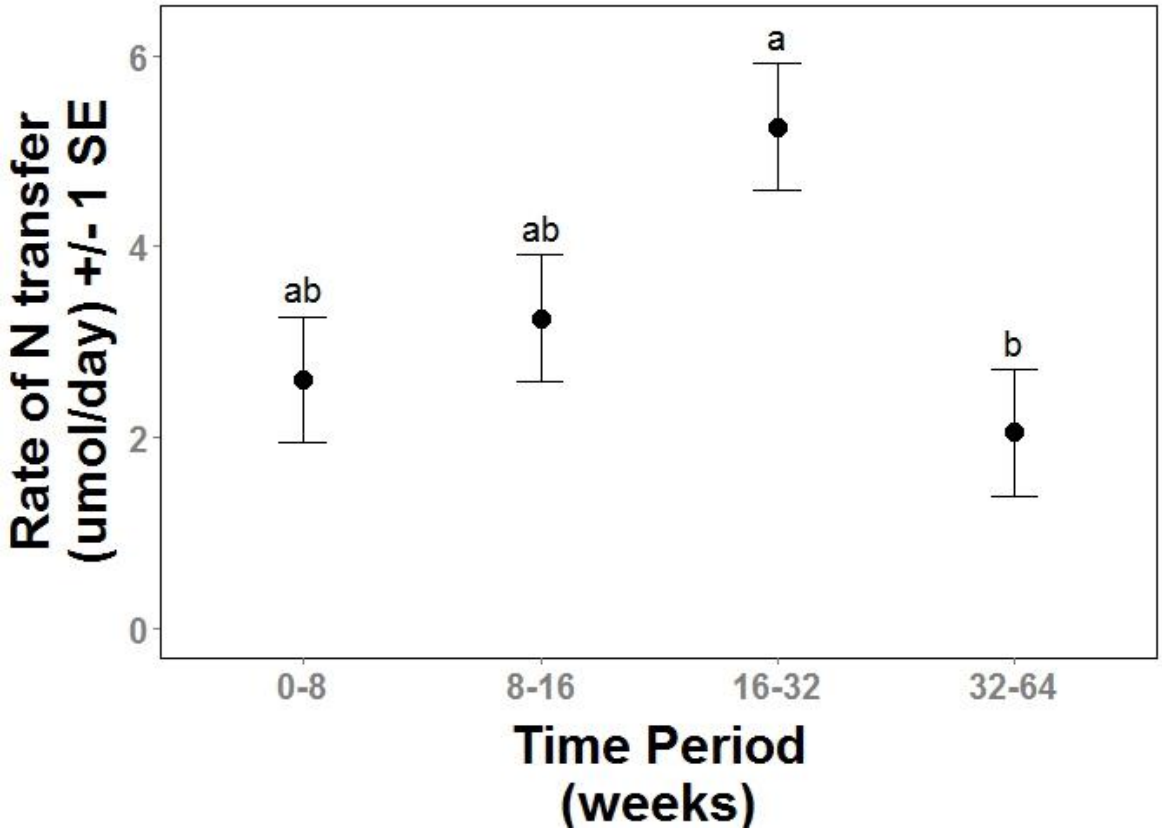

Figure 8: Rate of nitrogen transfer to seedlings. The rate of nitrogen transfer from *Rhizopogon occidentalis* to *Pinus radiata* seedlings varied significantly over time. Marginal means and standard errors shown.

Rate of phosphorus transfer to *P. radiata* seedlings from *R. occidentalis*

The rate of P transfer from *R. occidentalis* to *P. radiata* seedlings varied significantly over the time course of the mutualism ($F_{3,8}$=8.819, p=0.006). The rate of P transfer increased significantly over the first 32 weeks of the mutualism, followed by a significant reduction in the P transfer rate during the final time period (Figure 9). The rate of P transfer was not influenced by the population of the pine seedling ($F_{1,8}$=3.461, p=0.100). The pattern of P transfer rates over time tended to depend on pine population, with Cedros Island seedlings having a more consistent rate of P transfer over time, but this trend was not statistically significant at an alpha level of 0.05 (time period x population interaction: $F_{3,8}$=3.320, p=0.078; Figure 10).

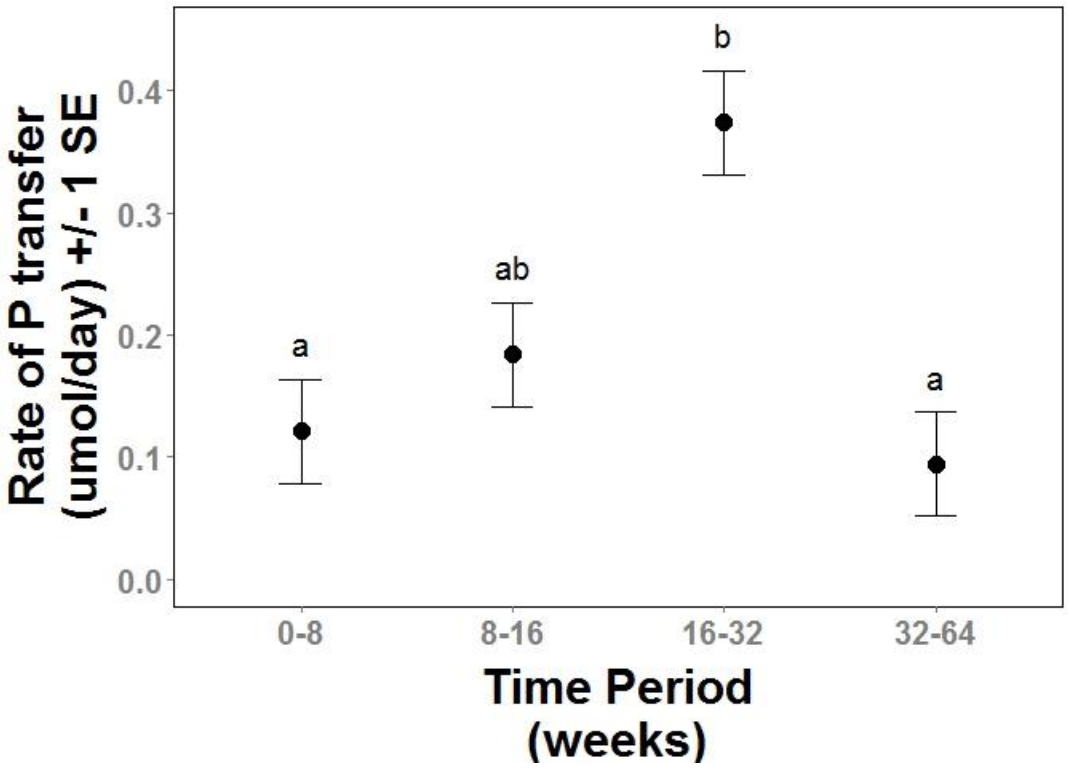

Figure 9: Rate of phosphorus transfer to seedlings. The rate of phosphorus transfer from *Rhizopogon occidentalis* to *Pinus radiata* seedlings varied significantly over time but not with pine population. Marginal means and standard errors shown.

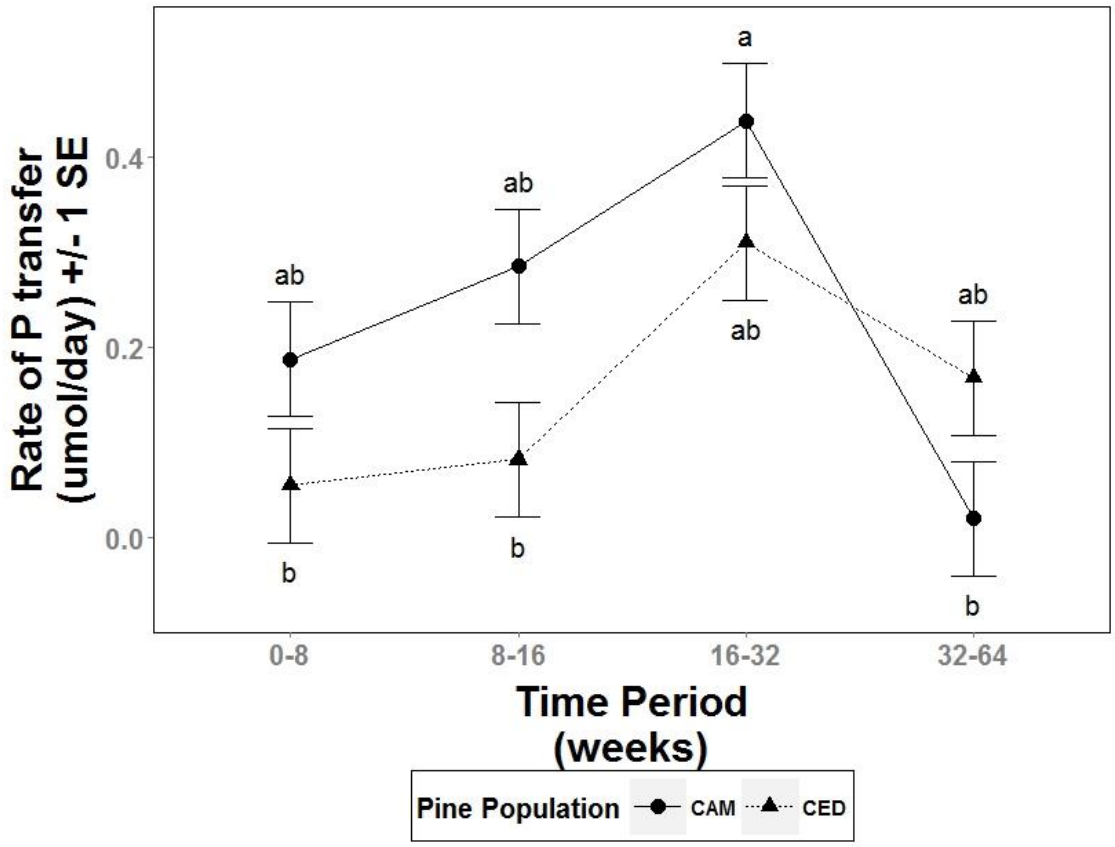

Figure 10: Patterns of phosphorus transfer rate for pine populations. The pattern of P transfer rates from *Rhizopogon occidentalis* to seedlings of *Pinus radiata* over time tended to depend on pine population, but was not statistically significant. Marginal means and standard errors shown. CAM = Cambria population and CED = Cedros Island population of *Pinus radiata*.

Rates of carbon transfer to *R. occidentalis* from *P. radiata* seedlings

The rate of C transferred to *R. occidentalis* and converted to fungal biomass increased significantly over the first 32 weeks of the mutualism ($F_{3,8}$=4.778, p=0.034, Figure 11). However, the rate of C transfer to fungal biomass was not influenced by the population of the pine seedling ($F_{1,8}$=2.018, p=0.193) and the pattern of C transfer rates to fungal biomass over time did not depend on pine population (time period x population interaction: $F_{3,8}$=0.593, p=0.637).

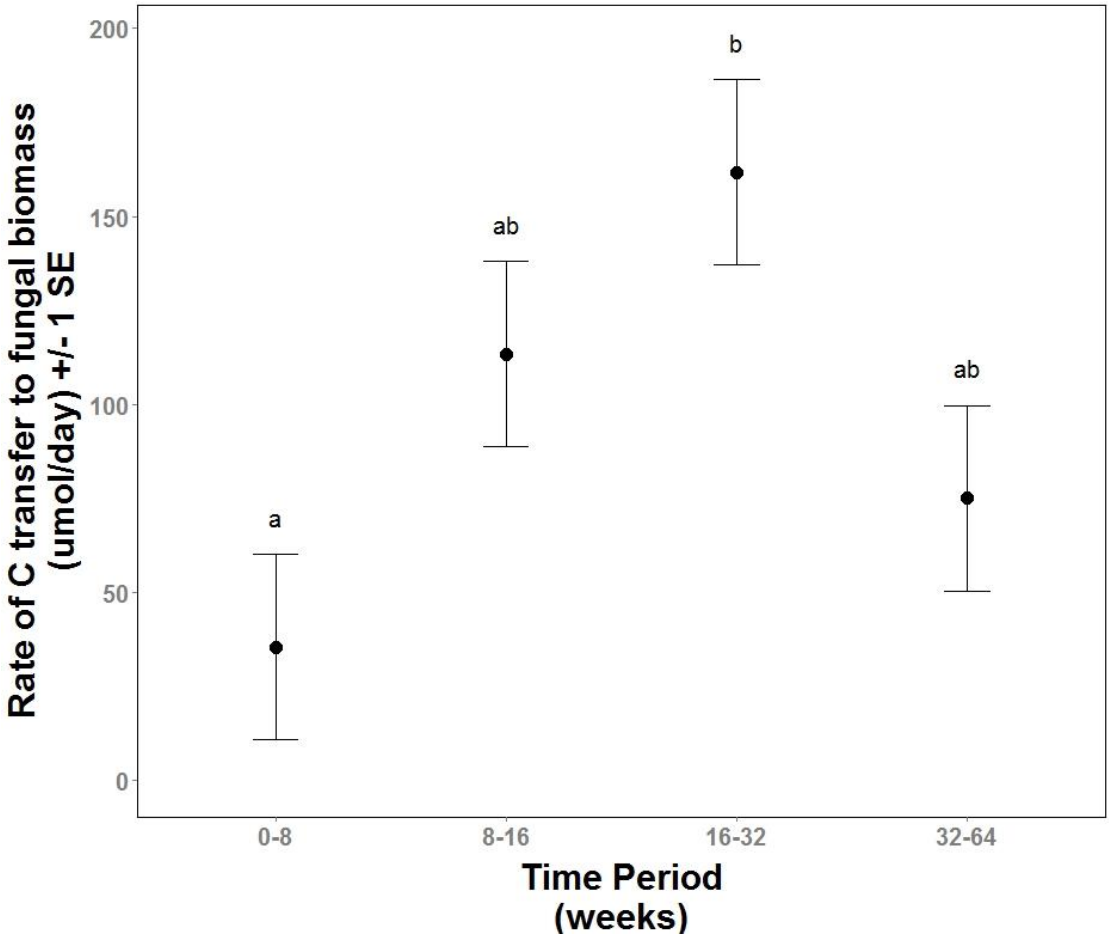

Figure 11: Rate of carbon transfer to fungal biomass. The rate of C transferred from *Pinus radiata* seedlings to *Rhizopogon occidentalis* and converted to fungal biomass increased significantly over the first 32 weeks of the mutualism. Marginal means and standard errors shown.

The rate of C transferred to *R. occidentalis* and released as fungal respiration increased significantly over time ($F_{3,8}$=7.595, p=0.010; Figure 12a). *Rhizopogon occidentalis* associated with seedlings from the Cambria population tended to have greater rates of C transfer released as fungal respiration; however, this trend was not statistically significant at an alpha level of 0.05 ($F_{1,8}$=4.414, p=0.069; Figure 12b). The variation over time in rates of C transfer released as fungal respiration did not depend on pine population (time period x population interaction: $F_{3,8}$=0.730, p=0.562).

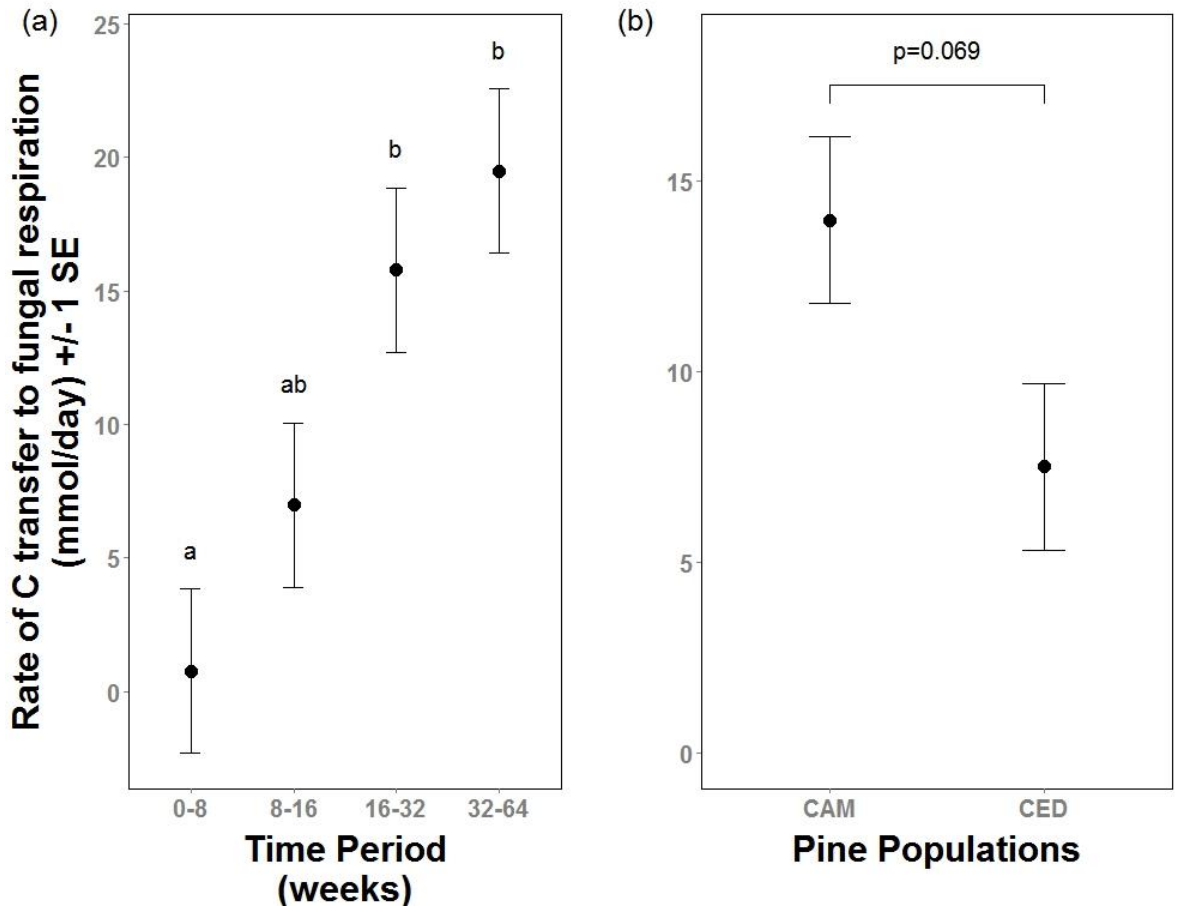

Figure 12: Rate of carbon transfer to fungal respiration. (a) The rate of carbon transferred from *Pinus radiata* seedlings to fungal respiration by *Rhizopogon occidentalis* varied significantly over time; (b) seedlings from the Cambria population tended to have higher rates of carbon transferred to fungal respiration than seedlings from the Cedros Island population. Marginal means and standard errors shown.

Similar results were found in the rate of total C transfer to *R. occidentalis*, which was driven by rates of C transferred to fungal respiration, since average rates of C transferred to fungal respiration were 100 times the average rate of C converted to fungal biomass. The rate of total C transferred to the fungus increased consistently over time, with a significant increase between the first and third time periods ($F_{3,8}$=7.538, p=0.010; Figure 13a). *Rhizopogon occidentalis* associated with seedlings from the Cambria population tended to have greater rates of C transfer; however, this trend was not

statistically significant at an alpha level of 0.05 ($F_{1,8}$=4.400, p=0.069). The pattern of C transfer rate over time did not depend on pine population (time period x population: $F_{3,8}$=0.729, p=0.563).

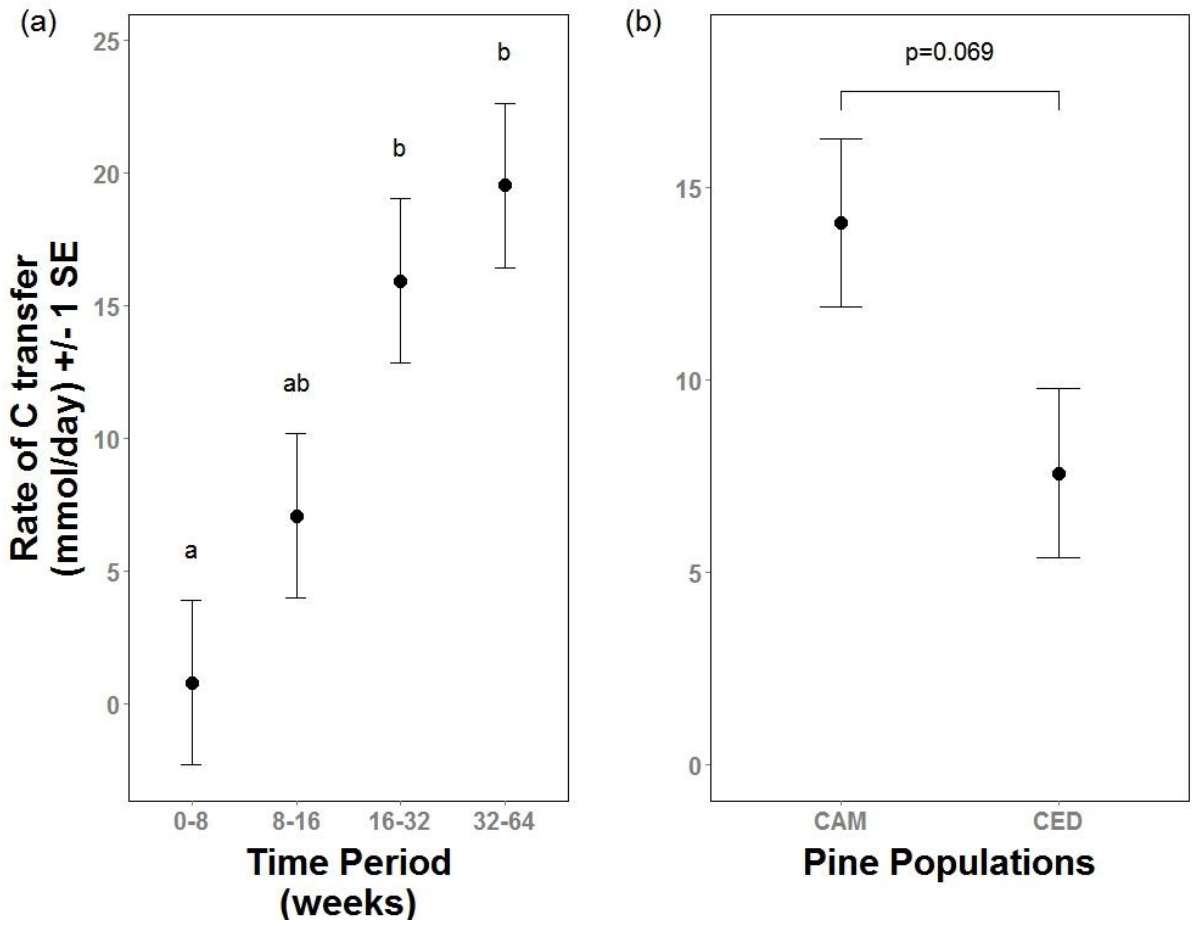

Figure 13: Rate of carbon transfer to fungus. (a) The rate of carbon transferred from *Pinus radiata* seedlings to the fungus *Rhizopogon occidentalis* increased significantly over time; (b) seedlings from the Cambria population tended to have higher rates of carbon transferred to the fungus than seedlings from the Cedros Island population. Marginal means and standard errors shown.

Resource exchange ratios

Because N, P, and C transfers followed similar patterns over time, the C:N exchange ratios did not vary significantly with time or with pine population (time period main effect: $F_{3,8}$=1.084, p=0.410; pine population main effect: $F_{1,8}$=1.274, p=0.292; time period x population interaction: $F_{3,8}$=0.896, p=0.484; $\bar{x}$=5348.5 ± 330.1). The C:P exchange ratios did not vary significantly with time or with the population of pine (time period main effect: $F_{3,8}$=0.339, p=0.798; pine population main effect: $F_{1,8}$=1.343, p=0.280; time period x population interaction: $F_{3,8}$=0.561, p=0.655; $\bar{x}$=132984.6 ± 13478.3).

Gross nutrient fluxes and gross resource exchange ratios

The gross fluxes of N, P, and C over the entire course of the experiment showed some interesting patterns (Figure 14). In particular, *R. occidentalis* associating with pine seedlings from the Cambria 9 family acquired more C over the course of the experiment, while returning a smaller amount of N compared to all other families of pine seedlings. This combination of results drives the pattern seen in the gross C:N and C:P exchange ratios, where seedlings from the Cambria 9 population established higher gross exchange ratios than seedlings from other genetic backgrounds (Figure 15).

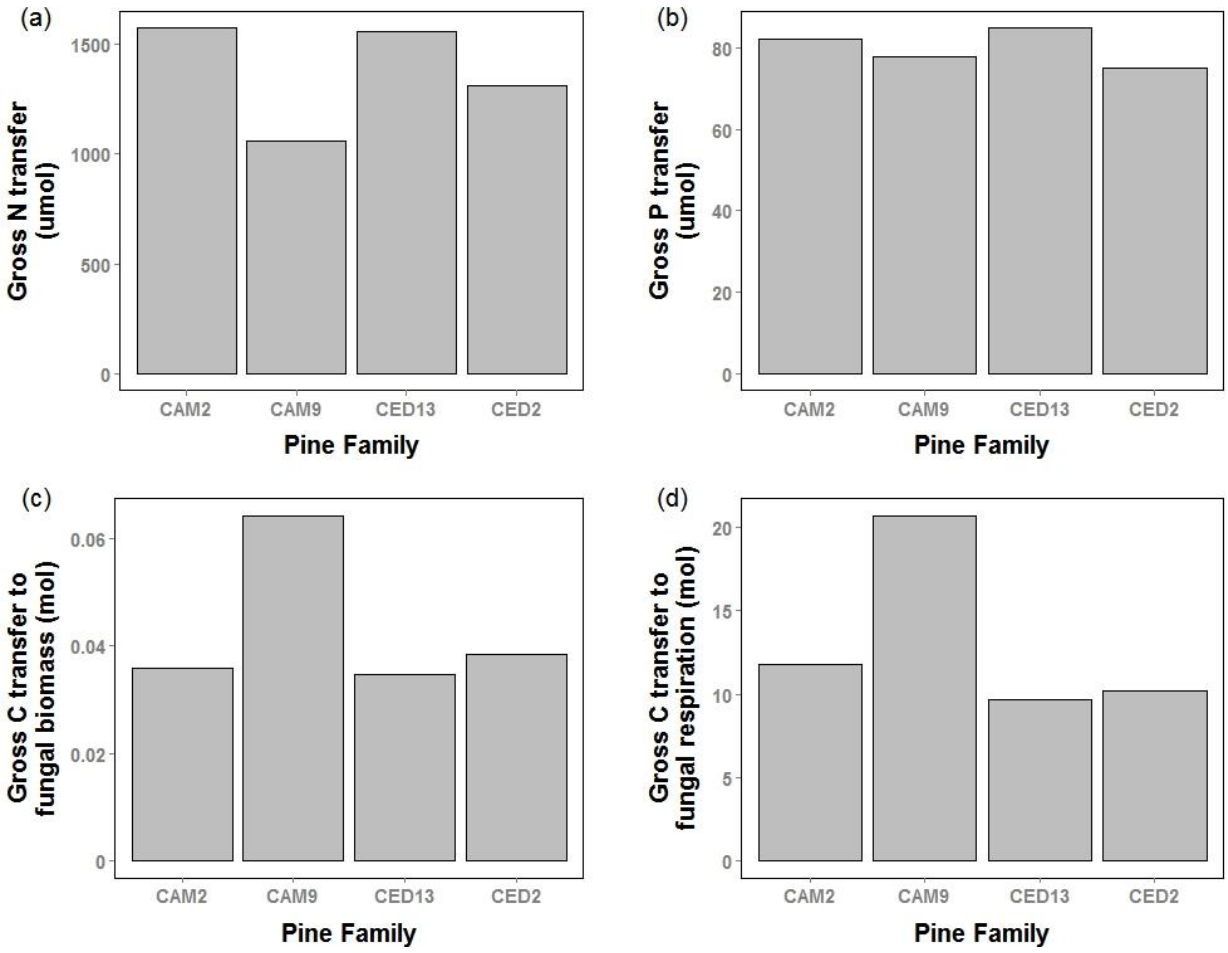

Figure 14: Gross resource transfer for pine families. Total nutrient transfers between *Pinus radiata* seedlings and the fungus *Rhizopogon occidentalis* over the course of the experiment; (a) total nitrogen transfer for each genetic family; (b) total phosphorus transfer for each genetic family; (c) total carbon transfer to fungal biomass for each genetic family; (d) total carbon transfer to fungal respiration for each genetic family.

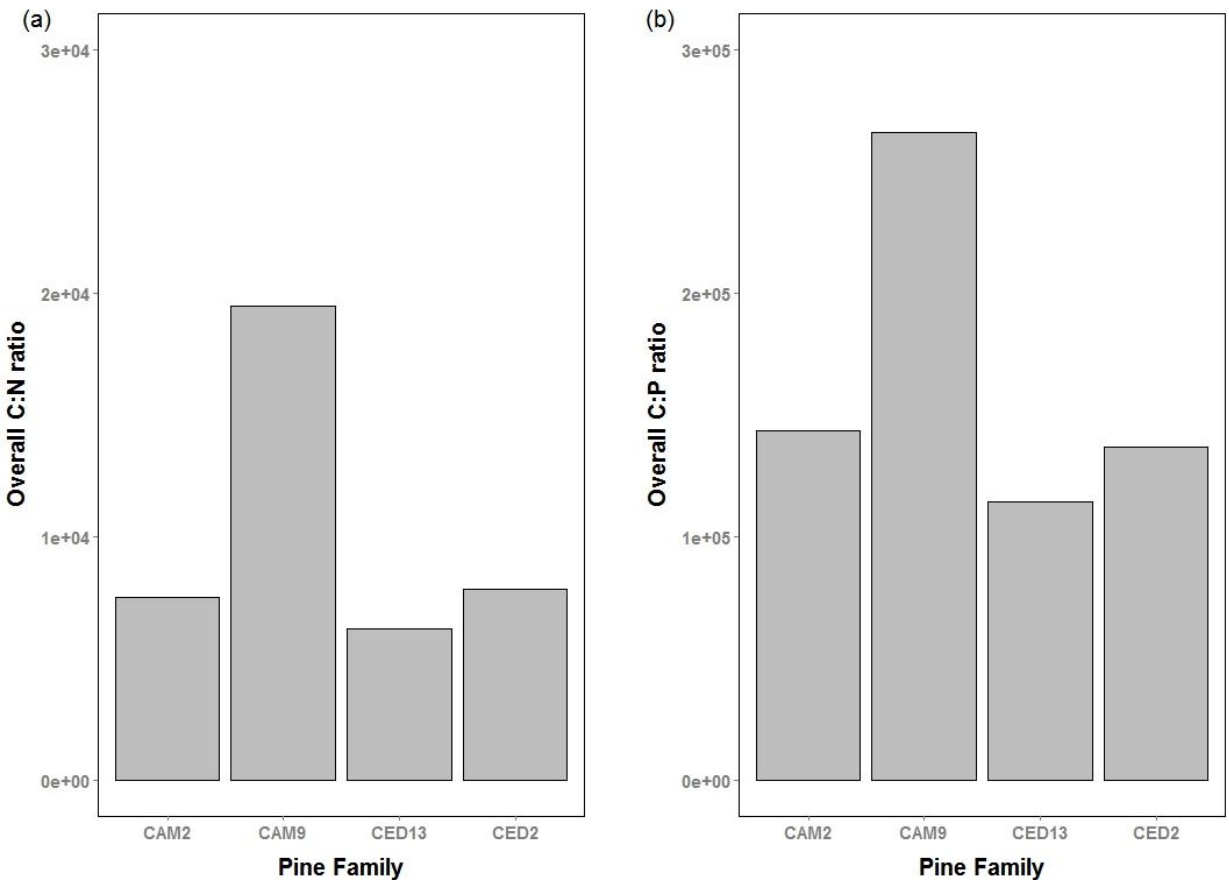

Figure 15: Gross resource exchange ratios for pine families. Pine seedlings from the Cambria 9 family of *Pinus radiata* established higher exchange ratios with *Rhizopogon occidentalis* than seedlings from other genetic backgrounds.

DISCUSSION

Was resource exchange with the ectomycorrhizal fungus R. occidentalis influenced by genetic variation in P. radiata?

Pinus radiata seedlings from the Cedros Island population accumulated less N and P, and transferred less C to the ectomycorrhizal fungus, *R. occidentalis*, compared to Cambria seedlings (Figures 4, 5, 13). Although N and P contents of seedlings depended on their population of origin, the rates of N and P transfer to the seedlings did not differ significantly between the two populations (Figures 8, 9). This may have been due to lower power in statistical tests for transfer rates caused by necessary averaging across replicates. Although *R. occidentalis* experienced a similar rate of C transfer to fungal biomass when associated with seedlings from either population (Figure 11), the fungus also experienced a reduced rate of C transfer to fungal respiration when associated with Cedros Island seedlings (Figure 12). This result indicates that *R. occidentalis* biomass was less metabolically active when in association with Cedros Island seedlings. These divergences in resource exchange dynamics between pines from the Cambria and Cedros Island populations may be related to the evolution of reduced compatibility with *R. occidentalis* seen previously in the Cedros Island population (Hoeksema and Thompson 2007). The dry, unproductive desert habitat of Cedros Island may select against C-costly ectomycorrhizal associations by pines with high biomass fungi like *R. occidentalis*. Reduced overall exchange of resources with *R. occidentalis* could have evolved as a strategy for *P. radiata* to prevent disadvantageous mycorrhizal associations. Such patterns of discrimination

against less-compatible mycorrhizal associates have been observed in whole plant and root organ culture manipulations of arbuscular mycorrhizal symbioses, where both plant and fungal partners are able to reward (with greater transfer of C) cooperative associates that supply greater amounts of nutrient resources (Bever et al. 2009, Kiers et al. 2011).

Although absolute resource fluxes varied between populations of *P. radiata*, C:N and C:P exchange ratios did not, due to consistent patterns of relative fluxes of C, N, and P for each pine population. Constant resource exchange ratios between populations indicates that the supply of C by the plant is proportional to the supply of N and P by the fungus (e.g. N or P gain by the plant). These results are supported by research indicating that roots supplying the host plant with greater amounts of P are rewarded with greater amounts of C (Kiers et al. 2011). My hypothesis that less favorable resource exchange ratios (i.e., greater amounts of C traded for units of N and P) would be established between *R. occidentalis* and Cedros Island seedlings was thus not supported with this experiment, since similar resource exchange ratios were established for both pine populations. Instead, my research suggests that total fluxes of nutrients are more important in determining the compatibility between mycorrhizal partners.

Within the Cambria population, there was apparently genetic variation in gross resource exchange ratios established in the *P. radiata*-*R. occidentalis* mycorrhizal mutualism, as seedlings from the Cambria 9 population transferred more C to mycorrhizal fungi than Cambria 2 seedlings, while receiving less N and P in return, resulting in greater resource exchange ratios (Figures 14, 15). The resource exchange dynamics of the Cambria 9 population support previous research indicating that C transfer to the fungus increases with reduced N uptake by the plant (Corrêa et al. 2008). Within-population variation in mycorrhizal traits has been documented previously in pines and indicates that the raw material for natural selection to act on is present and may lead to further evolutionary

changes in resource exchange traits (Piculell et al. 2008). Here, the potential exists for further evolution of resource exchange ratios within the Cambria population of *P. radiata*.

Did resource exchange between the ectomycorrhizal fungus R. occidentalis and seedlings of P. radiata vary over time?

Resource (C, N, and P) transfer rates increased over time and in a similar pattern to one another. The increasing rates of transfer of N, P, and C during the first 32 weeks of the mutualism indicate that an active and productive mutualism was established between the pine seedling and ectomycorrhizal fungus (Figures 8, 9, 12). The following plateau in nutrient transfers during the latter 32 weeks of the mutualism indicates a corresponding plateau in plant and fungal growth. This temporal pattern could be due to the size limitations of the mycocosm boxes or nutrient limitations of the soil which, in turn, limited resource transfers.

Resource exchange ratios (C:N, C:P) were found to be consistent over time. This result is most likely due to a lack of statistical power, considering that resource transfer rates increase similarly to one another over time although at drastically different magnitudes, which should result in variation in exchange ratios over time. Nonetheless, consistent exchange ratios during each time period indicates that, over long periods of time, the supply of C by the plant is proportional to the supply of N and P by the fungus (e.g. N or P gain by the plant). This differs from the nutrient dynamics found in *P. pinaster* associating with *Pisolithus tinctorius*, where C supply from the plant was dependent on N demand, rather than N gain, of the plant during the first four months of the mutualism (Corrêa et al. 2008, 2011). My hypothesis that resource exchange ratios would vary with C demand by the fungus was thus not supported with this experiment, since resource exchange ratios did not vary significantly over time.

The patterns of temporal variation in resource exchange dynamics of pines found in this study contrast with those seen in ectomycorrhizal willows. Jones et al. (1991) found that the rate of P transfer between *Salix viminalis* seedlings and *Thelephora terrestris* remained constant at 0.25 μmol/day over a 98-day experiment, while the phosphorus acquisition efficiency (P:C exchange ratio) declined between the first and second halves of the experiment. This result indicates that the patterns of C and P transfer differ from one another, with belowground C transfer increasing over time. However, these measurements may not be indicative of C transfer to the mycorrhizal fungus, since in the experiment by Jones et al. (1991), the amount of C transferred to fungi was not partitioned from total belowground C transfer (including plant roots), and thus C flow to roots may be driving these patterns. In contrast, the constant resource exchange ratios but fluctuating resource fluxes over time found in my research indicate that resource exchange ratios may be fixed traits of fungal species, with the mycorrhizal partners increasing total nutrient transfers when supply and demand for the nutrients are greater.

Developing a method to measure resource exchange ratios of mycorrhizal mutualisms

With my research, I have successfully measured resource exchange ratios in a mycorrhizal system using a total resource tracking approach conducted using dual-chambered mycocosms. Tracking the total transfers of resources exchanged allowed for the calculation of temporally integrated resource exchange ratios over periods of weeks and months, rather than measuring these ratios using flux pulses during short periods of hours or days (which essentially generates a point estimate). In addition, using a mycocosm approach enabled me to partition out different fates of traded nutrients. Some interesting findings have developed from this approach, which add to our knowledge of resource dynamics of mycorrhizal mutualisms.

The resource exchange ratio calculated in my research differs from previous studies attempting to calculate resource exchange ratios. Jones et al. (1991) estimated an average of 1051.9 μmol C were traded per μmol P between willow saplings and their ectomycorrhizal fungi. Comparatively, the C:P exchange ratio calculated in my research, ~133,000 μmol C per μmol P, greatly exceeds that of Jones et al. (1991), despite their estimate theoretically overestimating the C:P exchange ratio by including all C transferred belowground, including that which is respired by or incorporated as biomass in plant roots. Resource exchange ratios calculated by Pearson and Jakobsen (1993) for several arbuscular mycorrhizal associations range from 0.004-10.2 units of C per unit of P. Kiers et al. (2011) estimated the C:P exchange ratio established in an arbuscular mycorrhizal association using radioactively-labeled nutrients, but did not include C transfer to fungal respiration. By taking into account the multiple fates of C transferred to mycorrhizal fungi, I have shown that these resource exchange ratios may be much larger than previously thought.

The magnitude of estimated C transfer to the fungus, while greater than previously estimated, is plausible, considering the net C income of the plant via photosynthesis. Recorded net photosynthetic rates of *Pinus radiata* range from 3.5-8.6 mmol C/day (Duñabeitia et al. 2004, Booth and Hoeksema 2010, Mitchell et al. 2014). However, because these measurements were taken under lower light levels of a mature forest understory or greenhouse, or under chronic drought conditions, these photosynthetic rates are most likely underestimations of the photosynthetic rates of the pine seedlings in the present experiment. Hence, my estimation of ~10 mmol C/ day transferred to mycorrhizal fungi is plausible under the high light and water conditions of this experiment.

Additionally, the instantaneous rates of fungal respiration measured in this experiment fall within the wide range of those measured in previous studies. Using an aboveground CO_2 efflux method, I found an average instantaneous fungal respiration rate of 4.28×10^{-4} μmol CO_2/s/μg

ergosterol. When using methods similar to that of my experiment, the instantaneous fungal respiration rate of *Hebeloma crustuliniforme* associated with pine seedlings for 22 days was found to be ~112 μmol CO_2/s/μg ergosterol (Andersen and Rygiewicz 1995). Studies using in-soil CO_2 traps have measured instantaneous fungal respiration rates of ~$3.15x10^{-8}$ μmol CO_2/s/μg ergosterol for *Rhizopogon sp.* associated with pine seedlings and ~$5.5x10^{-7}$ μmol CO_2/s/μg ergosterol for *Paxillus involutus* associated with birch seedlings (Ek 1997, Bidartondo et al. 2001). The large difference in fungal respiration rates between these two measurement methods may be due to an underestimation by the in-ground CO_2 trap measurements, resulting from CO_2 losses through diffusion out of the fungal compartment. My method of measuring aboveground CO_2 efflux from fungal respiration with multiple measurements over an extended period of time potentially provides a more realistic picture of C transfer to mycorrhizal fungi.

Finally, the methods developed in this experiment can be employed to test the hypotheses predicted by economic models of mutualisms. Future lines of research would investigate how resource exchange dynamics are influenced by fungal taxa that differ in functional traits, such as exploration biomass type, and by resource limitation for both mycorrhizal partners. Such studies would further elucidate whether C:N and C:P exchange ratios or total resource fluxes are the definitive variables in resource exchange mutualisms.

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